1: Jpn J Exp Med. 1990 Dec;60(6):343-9. 

# erbB-2 gene expression in colorectal cancer.

**Tsioulias GJ, Muto T, Morioka Y, Yamamoto T, Mori S.**

1st Department of Surgery, Faculty of Medicine, University of Tokyo, Japan

The expression of the erbB-2 gene product was studied immunohistochemica on 38 normal colonic mucosae, 14 adenomas and 44 colon cancers, with the of two anti-erbB-2 antibodies, a rabbit polyclonal antibody specific for the intracellular domain and a mouse monoclonal antibody specific for the extracellular domain. Normal mucosae and adenomas were not stained. Five cases (11%) of colon cancer were positive with the polyclonal anti-erbB-2 antibody, while only one case was positive with the monoclonal antibody. M of the positive cases were in Dukes C stage. The rare overexpression of the erbB-2 protein in colon cancer seems to indicate a minor role for the gene in colorectal tumorigenesis.

PMID: 1982887 [PubMed - indexed for MEDLINE]

1: Eur J Cancer. 1992;29A(1):170. 

# c-erbB-2/neu in colorectal carcinoma: a potential prognostic value?

**Kapitanovic S, Spaventi R, Kusic B, Pavelic K.**

Publication Types:
- Letter

PMID: 1359899 [PubMed - indexed for MEDLINE]

1: Surg Oncol. 1992 Feb;1(1):97-105. Related Articles, l

# Correlation of epidermal growth factor receptor and c-erbB2 oncogene product to known prognostic indicators of colorectal cancer.

**Kluftinger AM, Robinson BW, Quenville NF, Finley RJ, Davis NL.**

Department of Surgery, University of British Columbia, Vancouver, Canada.

Both epidermal growth factor receptor (EGFr) and the oncogene product of c erbB2 have been shown to be expressed by human malignancies, and in som cases to relate to clinical outcomes. This study investigates the correlation between the presence of these receptor proteins and known prognostic indica of colorectal cancer. Indirect immunoperoxidase staining of 32 freshly frozer surgical specimens revealed an overall expression of EGFr and c-erbB2 of 4: and 38%, respectively. A significantly higher rate of EGFr expression was found in tumours of more advanced stage (Dukes C and D), poor differentiat and those exhibiting vascular and lymphatic invasion. The presence of the c-erbB2 protein did not correlate with any of these variables. Expression of the molecules appeared to be independent and positive staining for both receptor occurred in only 19% of cases. EGFr may play a future role as a prognostic t in colorectal cancer.

PMID: 1285215 [PubMed - indexed for MEDLINE]

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1: J Clin Pathol. 1992 Aug;45(8):726-7. Related Articles, l

# HER2 (c-erbB-2) oncoprotein expression in colorectal adenocarcinoma: an immunohistological study using three different antibodies.

**Arnaout AH, Dawson PM, Soomro S, Taylor P, Theodorou NA, Feldma M, Fendly BM, Shepard HM, Shousha S.**

Department of Histopathology, Charing Cross Hospital, Charing Cross and Westminster Medical School, London.

Paraffin wax sections of 70 surgically resected colorectal adenocarcinomas w examined for the overexpression of HER2/c-erbB-2 oncoprotein using three different specific antibodies and the avidin-biotin immunoperoxidase techniq The patients included 38 men and 32 women aged between 47 and 80 years. The tumours were derived from various parts of the large intestinal tract, and represented all three stages of Dukes' classification and the three histological grades of differentiation. Many tumour sections also included adjacent norm or transitional mucosa. Eight tubular adenomas found in the colectomy specimens in association with some carcinomas were also examined. No positive membrane staining was seen in any of the 70 carcinomas, four adenomas, two hyperplastic polyps, nor in the adjacent normal or transitional mucosa. It is suggested that the overexpression of c-erbB-2 gene product is unlikely to be as common and as pronounced in colorectal adenocarcinoma a is in ductal carcinoma of the breast.

PMID: 1357006 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

1: J Surg Oncol. 1993 Dec;54(4):246-51. Related Articles, l

# Study of c-erbB-2 protein and epidermal growth factor receptor expression and DNA ploidy pattern in colorectal carcinoma.

**Nakae S, Shimada E, Urakawa T.**

Department of Surgery, Kobe Rosai Hospital of the Labour Welfare Corporation, Japan.

Correlation of c-erbB-2 protein (n = 44), epidermal growth factor receptor (EGFR) (n = 41) expression, and DNA ploidy pattern (n = 42) with clinical outcomes of human colorectal cancers was studied. Using monoclonal antibodies against c-erbB-2 protein and EGFR, an immunohistochemical stu of the expression of c-erbB-2 protein and EGFR in frozen tissue sections fro the lesion was performed. There was no significant correlation between the expression of c-erbB-2 protein and clinicopathological findings such as, tum size, histological type, depth of invasion, lymph node metastasis, lymphatic vessel invasion, or venous invasion. However, the incidence of c-erbB-2 pro expression in Dukes D was significantly higher (9/10, 90%) than that in Duk A to C (16/34, 47.1%). Similar tendency was also observed in the expression EGFR. Aneuploid case was observed in 12 of observed 25 (48%) cases with lymph node metastasis, while it was observed in 16 of 17 cases (94.1%) with lymph node metastasis and there was significant association between DNA ploidy pattern and lymph node metastasis ($P < 0.01$) and most of the cases (17/20, 85%) were aneuploidy in Dukes C and D. The results above suggest the expression of c-erbB-2 protein or EGFR was associated with distant metastasis, while on the other hand DNA ploidy pattern was correlated with lymph node metastasis.

PMID: 7902886 [PubMed - indexed for MEDLINE]

1: Cancer Detect Prev. 1994;18(2):97-101. 

# High c-erbB-2 protein level in colorectal adenocarcinomas correlates with clinical parameters.

**Kapitanovic S, Spaventi R, Poljak L, Kapitanovic M, Pavelic ZP, Gluckman JL, Spaventi S, Pavelic K.**

Department of Molecular Medicine, Ruder Boskovic Institute, Zagreb, Croat

Sections of normal colon, adenomas, and adenocarcinomas were examined b immunohistochemistry for the expression of c-erbB-2 proto-oncogene produ in order to assess its potential diagnostic value in predicting the malignant potential of these lesions. We compared the degree of epithelial abnormality clinical parameters, including Dukes' classification and survival time with th extent of immunoperoxidase staining. Sections of normal colon and tubular adenomas examined demonstrated a weak immunostaining localized to the luminal surface cells. However, higher level of c-erbB-2 expression was observed in dysplastic areas of one tubular and one villous adenoma. Out of adenocarcinomas, only 2 samples showed weak immunoreaction, while 38 samples were moderately or strongly positive for c-erbB-2 protein. The inten of staining correlated positively with the stage of disease and postoperative survival time.

PMID: 7912991 [PubMed - indexed for MEDLINE]

1: Anticancer Res. 1995 Nov-Dec;15(6B):2889-94. Related Articles, L

# Expression of growth factors and their receptors in human early colorectal carcinomas: immunohistochemical study.

**Shirai H, Ueno E, Osaki M, Tatebe S, Ito H, Kaibara N.**

First Department of Pathology, Faculty of Medicine, Tottori University, Japa

Human colorectal carcinomas have been demonstrated to express a variety of growth factors and their cognate receptors, forming multi-autocrine, juxtacrin and/or paracrine loops. Little information, however, is available on their expression in early colorectal carcinomas in which two genetic pathways exist i.e. adenoma-carcinoma sequence and de novo carcinoma. This study was conducted in a total of 68 early colorectal carcinomas invading the submucosa which were subdivided into two categories by the presence of adenomatous components, namely (a) 38 carcinomas with an adenomatous component and 30 carcinoma without an adenomatous component. The tumous were also classified as polypoid, flat elevated and flat depressed type. Formalin-fixed, paraffinembedded specimens were immunostained for epidermal growth factor (EGF), transforming growth factor alpha(TGFalpha), cripto, EGF-receptor (EGFR) and c-ERBB2 gene product. Of the 68 early colorectal carcinomas, EGF, TGF-alpha, cripto, EGFR and c-ERBB2 products were detected at various degrees 24(35%), 50(74%), 31(46%), 11(16%), and 34(50%), respectively. The expression was compared between 35 polypoid carcinomas with an adenoma component (suitable for adenoma-carcinoma sequence), 14 flat carcinomas without an adenoma component (possible de novo carcinomas). A significantly higher incidence ($P < 0.05$) of expression of the following was noted; TGF-alpha in the polypoid carcinomas with an adenoma component, and EGF and ERBB2 gene product in the carcinomas without an adenoma component. There was no significant difference in the incidence of cripto and EGFR, implying common events between two categories. These results indicate that two pathways exist in tumourigenesis, in which the growth factors and their receptors are expressed in different manners. TGF-alpha might play a crucial role in carcinomas arising from adenoma, while EGF and c-ERBB2 gene products are strongly indicative of de novo carcinomas.

PMID: 8669884 [PubMed - indexed for MEDLINE]

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Nov 18 2003 07:

1: Jpn J Clin Oncol. 1995 Dec;25(6):240-9. Related Articles, l

# Association of epidermal growth factor-related peptides and typ receptor tyrosine kinase receptors with prognosis of human colorectal carcinomas.

**Saeki T, Salomon DS, Johnson GR, Gullick WJ, Mandai K, Yamagami I Moriwaki S, Tanada M, Takashima S, Tahara E.**

Department of Clinical Research, Pathology and Surgery, National Shikoku Cancer Center Hospital, Matsuyama.

The frequency of expression and localization of cripto-1 (CR-1), amphiregul (AR), transforming growth factor alpha (TGF alpha), epidermal growth facto receptor (EGFR) and erbB-2 were examined by immunohistochemistry in 45 carcinomas and adjacent non-involved normal colon mucosa. Thirty (66.7%) (53.3%), 23 (51.1%), 23 (51.1%) and 13 (28.9%) of the 45 carcinomas show positive staining for CR-1, AR, TGF alpha, EGFR and erbB-2, respectively, whereas 7 (15.5%), 17 (37.7%), 15 (33.3%), 20 (44.4%) and 0 (0%) of the corresponding non-involved normal mucosa specimens were reactive. Amon 13 carcinomas with lymph node involvement, 10 (76.9%), 8 (61.5%), 10 (76.9%), 8 (61.5%) and 7 (53.8%) exhibited positive staining for CR-1, AR, TGF-alpha, EGFR and erbB-2, respectively. There was a statistically signific association between the frequency of either TGF alpha ($P < 0.05$) or erbB-2 ( 0.05) expression and lymph node metastasis. In addition, a significantly high frequency of positive staining for TGF alpha was observed in Dukes' grade C carcinomas ($P < 0.05$). Finally, significant trends for coexpression of EGFR : either TGF alpha ($P < 0.01$) or AR ($P < 0.05$) were detected in carcinomas. These data suggest that AR and TGF alpha may play an important role in the development of colorectal carcinomas through an autocrine mechanism involving EGFR, and demonstrate that TGF alpha and erbB-2 may be more reliable indicators of metastasis or prognosis than CR-1, AR or EGFR in hun colon cancers.

PMID: 8523820 [PubMed - indexed for MEDLINE]

Nov 18 2003 07:

1: Oncogene. 1999 Jan 14;18(2):305-14. Related Articles, l

nature publishing group

# Regulation of cyclooxygenase-2 pathway by HER2 receptor.

**Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar**

Department of Clinical Investigation, The University of Texas MD Anderson Cancer Center, Houston 77030, USA.

Emerging lines of evidence suggest that in addition to growth factors, the process of colorectal tumorigenesis may also be driven by the upregulation o the inducible form of cyclooxygenase-2 (COX-2), an enzyme responsible for the conversion of arachidonic acid to PGEs. The present study was undertake to investigate the expression and activation of the HER family members, and explore the regulation of COX-2 expression by the HER2 pathway in human colorectal cancer cells. Here, we report that human colorectal cancer cell line express abundant levels of HER2 and HER3 receptors, and are growth-stimulated by recombinant neu-differentiation factor-beta 1 (NDF). NDF-treatment of colorectal cancer cells was accompanied by increased tyrosine phosphorylation and heterodimerization of HER3 with HER2. In addition, w demonstrated that HER2 and HER3 receptors in colorectal cancer cells are constitutively phosphorylated on tyrosine residues and form heterodimeric complexes in the absence of exogenous NDF. Inhibition of HER2/HER3 signaling by an anti-HER3 mAb against the ligand binding site resulted in a decrease in the levels of constitutively activated HER2/ HER3 heterodimers, and the unexpected reduction of COX-2 expression. Activation of the HER2/HER3 pathway by NDF induced the activation of COX-2 promoter, expression of COX-2 mRNA, COX-2 protein and accumulation of prostaglandin E2 in the culture medium. Finally, we demonstrated that NDF promotes the ability of colorectal cancer cells to survive in an extracellular matrix milieu, such as Matrigel, and also to invade through a 8 microm poro membrane. These biological activities of NDF and its stimulation of cell proliferation are blocked by a specific inhibitor of COX-2. Taken together, o findings provide the first biochemical evidence of a possible role of the COX pathway in the mitogenic action of NDF in colorectal cancer cells where it m be constitutively upregulated due to the autocrine/paracrine activation of HE HER3 heterodimers.

PMID: 9927187 [PubMed - indexed for MEDLINE]

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Nov 18 2003 07:

1: Hum Pathol. 1998 Aug;29(8):771-7. Related Articles, l

# Increased expression of erbB3 in colorectal cancer is associated with concomitant increase in the level of erbB2.

**Maurer CA, Friess H, Kretschmann B, Zimmermann A, Stauffer A, Bae HU, Korc M, Buchler MW.**

Department of Visceral and Transplantation Surgery, University of Berne, Inselpital, Switzerland.

ErbB3 is a transmembrane signaling molecule that shares close structural homology with epidermal growth factor receptor (EGFR), erbB2, and erbB4. They have all been implicated in cell transformation and tumor pathogenesis, but very little is known about the role of erbB3 in normal colon and colorecta cancer. Therefore, in the current study, we determined whether erbB3 is foun in normal human colon and whether its expression is altered in colorectal cancer. Because of some evidence that erbB3 might interact with erbB2 and EGFR, respectively, by heterodimerization, we also included erbB2 and EGF analysis with special regard to coexpression. The study was performed on 35 patients operated on for colorectal carcinoma. The normal human colon show weak erbB3 and erbB2 immunostaining, predominantly in surface epithelial cells. EGFR immunoreactivity in normal colon varied from weak to strong. I contrast, in 31 of 35 (89%) and in 29 of 35 (83%) colonic cancers, moderate strong immunoreactivity for erbB3 and erbB2, respectively, was present in m epithelial cancer cells. A concomitant erbB3 and erbB2 immunostaining advantage could be found in 77% of cancerous tissues in comparison with th normal colon. No difference in EGFR immunostaining was evident between normal colon and cancer. Northern blot analysis showed an increase in erbB3 and erbB2 mRNA levels in 64% of cancers in comparison with normal colon samples. By densitometry, 2.3-fold and a 1.5-fold significant increases in erb and erbB2 mRNA levels, respectively, were calculated in the cancerous tissu Eighty-five percent of cancers with erbB3 mRNA overexpression showed an increase in erbB2 mRNA. Southern blot analysis did not indicate any gene amplification or rearrangement responsible for erbB2 or erbB3 overexpressic EGFR, however, was decreased in cancer on mRNA level. These findings indicate that erbB2 and erbB3, but not EGFR, may contribute to tumor growt and disease progression in colon cancer. The correlation between increased erbB2 and erbB3 expression in both Northern blots and immunohistochemica analysis suggests a co-overexpression of erbB2 and erbB3 and might support

the hypothesis that these two growth factor receptors act together by heterodimer formation.

PMID: 9712416 [PubMed - indexed for MEDLINE]

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| 19 | 72 | heterodimeriz$5 and (her2 or cerbb2 or erbb2) | USPAT | 2003/04/29 12:24 |
| 20 | 20442 | (colon or colorectal or rectal) and (cancer or carcinoma or tumor or neoplasm) | USPAT | 2003/11/20 11:44 |
| 21 | 4568 | her2 or cerbb2 or erbb2 or p185 or neu or p185neu or erbb2p185 or p185erbb2 or erbb2neu | USPAT | 2003/11/20 11:42 |
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| 23 | 1936 | 424/130.1,133.1,134.1,135.1,136.1,138.1,141.1,142.1,143.1,152.1,155.1,156.1,172.1,174.1.ccls. | USPAT | 2003/11/20 11:43 |
| 24 | 87 | (((colon or colorectal or rectal) and (cancer or carcinoma or tumor or neoplasm)) and (her2 or cerbb2 or erbb2 or p185 or neu or p185neu or erbb2p185 or p185erbb2 or erbb2neu)) and 424/130.1,133.1,134.1,135.1,136.1,138.1,141.1,142.1,143.1,152.1,155.1,156.1,172.1,174.1.ccls. | USPAT | 2003/11/20 11:46 |
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| 28 | 87 | (((colon or colorectal or rectal) and (cancer or carcinoma or tumor or neoplasm)) and (her2 or cerbb2 or erbb2 or p185 or neu or p185neu or erbb2p185 or p185erbb2 or erbb2neu)) and 424/130.1,133.1,134.1,135.1,136.1,138.1,141.1,142.1,143.1,152.1,155.1,156.1,172.1,174.1.ccls. | USPAT | 2003/11/20 12:05 |
| 29 | 297 | colorectal.clm. | USPAT | 2003/11/20 12:05 |
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| 31 | 2954 | lung.clm. | USPAT | 2003/11/20 12:08 |
| 32 | 92 | 424/130.1,133.1,134.1,135.1,136.1,138.1,141.1,142.1,143.1,152.1,155.1,156.1,172.1,174.1.ccls. and lung.clm. | USPAT | 2003/11/20 12:08 |

# DIAGNOSTIC PROTOCOL

## CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation of copending U.S. application Ser. No. 08/922,873, filed Sep. 3, 1997, entitled "Diagnostic Protocol."

## FIELD OF THE INVENTION

The present invention relates to a diagnostic protocol incorporating a medical prognostic; the prognostic is designed to predict the survival of a given patient, in whom malignancy has been previously diagnosed.

## BACKGROUND OF THE INVENTION

The field of pathology is evolving so as to embrace two disciplines, one of which is subsumed into the other: diagnosis and prognosis. Each is a medical discipline in which biochemistry and molecular biology play increasingly important roles, allowing innovations in traditional diagnosis as well as reliable protocols for prospective outcome prediction. If a diagnostic test or assay, or "diagnostic," provides specific information regarding the nature of a disease, a "prognostic" test or assay provides specific information regarding the outcome of that disease—but within the conceptual confines of the original diagnostic. Properly developed prognostics allow a determination to be made in advance, with statistically significant and surprising accuracy and precision, whether a given drug or active agent, or therapeutic protocol including surgery or other treatments, will be effective to inhibit or to overcome the disease or condition in question.

In a very real sense, prognostics give a wider scope of benefit than traditional diagnostics do, even though they fall within the diagnostic field in general. Before the advent of the field of prognostics, various therapies had to be attempted sequentially based upon general guidelines developed from overall patient population data, but in many cases these general guidelines had disappointing predictive value with respect to any given patient or any given treatment. Against this backdrop, it can be seen that a prognostic can yield not only scientific and medical value but also both a heretofore-elusive humanitarian advantage (quality of life) as well as significant economic benefit (cost effectiveness). With a prognostic, for example, a given patient need not endure a given treatment simply to ascertain whether that treatment is likely to be effective. A properly designed prognostic gives a health care provider information about risk category and likelihood of survival, which in turn assists in determining appropriate therapy. It is easy to appreciate the particular utility of a prognostic in the challenging area of cancer treatment, where the benefit of a patient's not having to endure unnecessary treatment may be the greatest for any disease.

Cost savings also become significant when medical practitioners are provided with a tool by which to predict, for a given patient, whether a given therapy will be effective—because therapies unlikely to be effective are generally passed over at the outset. (Alternatively, the prognostic's predictive utility may appropriately identify patients for inclusion in the prospective investigation of novel treatments.) When therapies unlikely to be effective are skipped altogether, the costs (and waste) involved in the failed therapy are also avoided. Neither the cost nor the health benefit of avoiding likely-to-be-futile therapies should be underestimated. The improvement in morale in any patient who knows his chosen therapy is predictably



effective as to him or her individually itself contributes to the successful therapy in a manner analogous to the placebo effect.

In general, the best prognostics are those in which the particular biochemistry or molecular biology of biopsied tissue—or, alternatively, of blood or body fluids—can be assayed and quantitated to yield an objective outcome likelihood. Such biochemical "markers" might be anything, including but not limited to catabolytes, anabolytes, enzymes, hormones, other expressed peptides or proteins, distinct saccharides, or any other distinctive biomolecule. As a design consideration, the theoretically best biochemical marker for a prognostic would be one or more uniquely expressed peptides or proteins, because these could be readily identified (and quantitated) by corresponding monoclonal antibodies. The ideal cancer prognostic would therefore involve the identification of a critical threshold expression level for one or more unique peptides or proteins having prognostic significance. Such an assay could be performed with existing laboratory reagents and equipment, using standard monoclonal antibodies and optical-counting quantitation techniques, and would therefore be inexpensive as well as accurate and precise. Because the assay would be undertaken to ascertain expression levels in a given patient, the results would have prognostic value specific to that patient.

## SUMMARY OF THE INVENTION

In order to meet this need for optimal, risk-adjusted clinical decisions, the present invention is a cancer prognostic having particular utility in the prognosis of head and neck squamous cell cancer, in which the expression levels of either or both of Transforming Growth Factor Alpha (TGF-α) or Epidermal Growth Factor Receptor (EGFR) are assayed directly and separately from a sample of tumor tissue. The expression level once quantitated is normalized as to standard, and the standardized expression level is compared to the prognostic threshold of about 83% of standard for TGF-α or of about 23% of standard for EGFR, or the corresponding upper threshold of the "low" tertile regardless of how calculated. Virtually "all" (if not absolutely all) patients demonstrating such low expression level survive at least five years after initial diagnosis, assuming completion of treatment with standard surgical tumor excision and radiation protocols for squamous cell head and neck cancer. Whether an individual patient's expression levels of TGF-α and EGFR fall inside or outside of this category signifies whether the patient is in a good or poor prognostic category, respectively, which in turn guides appropriate choice of therapy.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** is a Table illustrating TGF-α and EGFR expression levels in a study of 91 patients.

FIG. **2** is a Table illustrating association of potential prognostic factors and disease free survival in the same study.

FIG. **3** is a Table illustrating association of potential prognostic factors and overall cause-specific survival in the same study.

FIG. **4** is a graph showing individual patients plotted against their elevated TGF-α and EGFR levels.

FIGS. ***5a*** and ***5b*** are graphs showing proportion of patients (divided into tertiles) surviving with no evidence of disease, over time.

FIGS. *6a* and *6b* are graphs illustrating overall cause-specific survival proportion over time for the patient tertiles.

FIGS. *7a, 7b, 7c* and *7d* are graphs illustrating survival proportion over time for the same patient tertiles divided as to their nodal staging, and further illustrates how the present prognostic changes the way patients are managed vis a vis the use of nodal staging alone.

## DETAILED DESCRIPTION OF THE INVENTION

Transforming Growth Factor Alpha (TGF-α) is a polypeptide of 50 amino acids. It was first isolated from a retrovirus-transformed mouse cell line and subsequently was identified in human tumor cells, in early rat embryo cells and in cell cultures from the human pituitary gland. Transforming Growth Factor Alpha (TGF-α) appears to be closely related to Epidermal Growth Factor (EGF), both structurally and functionally, and both bind to the same receptor, i.e., Epidermal Growth Factor Receptor (EGFR). Study of TGF-α has heretofore concentrated on its presence as a cancer marker and the likely value of TGF-α antagonists as therapeutic tools, not on quantitative expression levels of TGF-α or their potential prognostic significance.

Head and neck squamous cell carcinoma is an epithelial malignancy arising in the mucosa of the upper aerodigestive tract. Potential anatomic sites affected include the oral cavity, oropharynx, hypopharynx and larynx. Approximately half of the patients diagnosed have traditionally been cured of their initial tumor. Factors such as age, sex, tumor site, "TNM" stage and histologic grade have generally been relied on to assist in guiding treatment decisions but are not in fact useful predictors of outcome. Nodal stage assessment of malignancy is the best prior art predictor of survival, but the present prognostic is an even better predictor of outcome (see FIG. 7) and accordingly serves as an improved tool for guiding choice of therapy.

As a result of a study performed with respect to a statistically significant sample of 91 human patients, described further below, it was surprisingly found that literally all patients exhibiting an expression level of TGF-α/EGFR levels less than 83/23% of standard survived in excess of five years following standard surgical and radiation treatment for squamous cell head and neck cancer. A surprisingly high percentage of these furthermore survived with "No Evidence of Disease" (NED). These unexpected results in turn allowed the development of a prognostic assay wherein expression levels of either or both of Transforming Growth Factor Alpha (TGF-α) or Epidermal Growth Factor Receptor (EGFR) could be quantitated and normalized as to standard, and the standardized expression level is compared to the prognostic threshold of about 83/23% of standard. Because virtually all if not all patients demonstrating an expression level of 83/23% of standard or less will survive at least five ears after initial diagnosis, assuming standard treatment, such low expression levels of TGF-α and EGFR provide a prognostic indicating good patient prognosis with such standard therapy.

By contrast, and as illustrated vividly by the data summarized in this specification, if the patient demonstrates an expression level of greater than about 83/23% of standard, or the corresponding upper threshold of the "low" tertile regardless of how calculated, the patient can be seen to be in the poor prognosis category, and this information is valuable in guiding choice of therapy. It is not within the scope of the present prognostic assay to delineate actual choice of therapy, because the invention instead provides important information (risk category) to enable appropriate choice of therapy by the skilled practitioner. For purposes of illustration, however, appropriate choice of therapy for patients found to be in the poor prognostic category might include, without limitation, alternative surgical, radiation and/or chemotherapeutic treatments, monoclonal antibody therapy against EGFR such as those used in patients with lung squamous cell carcinoma, or fusion proteins or immunotoxins against TGF-α or EGFR using toxins elaborated by Pseudomonas or Diphtheria species, or other therapies yet to be developed. It is believed that the prognostic threshold identified herein moreover extrapolates to and applies to other cancers besides squamous cell head and neck cancer, based upon the previously documented expression of TGF-α by malignant epithelial cells of virtually or completely all types.

The study which led to the above conclusions was conducted as follows. Archival tissue samples (paraffin embedded) of 91 head and neck squamous cell carcinomas from patients undergoing resection for head and neck cancer from November 1990 to February 1993 were obtained from the diagnostic histopathology laboratories at the University of Pittsburgh Medical Center. Pertinent patient information was abstracted from the medical records. No patient had distant metastasis at the time of tumor resection.

All patients underwent complete surgical resection of the primary tumor with negative surgical margins and 84.6% (77/91) underwent dissection of the cervical lymph nodes with pathologic staging of the regional lymphatics (N-stage). Clinical staging was conducted according to accepted protocols, and clinical follow-up was available for all patients until October, 1996. Patients were classified according to disease status (alive without evidence of disease ("NED"), dead of disease, or dead of other causes). Statistical analysis was performed on the entire patient population.

Diagnosis of squamous cell carcinoma was based on conventional morphologic examination of paraffin-embedded specimens. Staining was performed on tissue sections using monoclonal antibodies specific for TGF-α and EGFR (available from Calbiochem/Oncogene Science and Genosys/Cambridge Research, respectively). A sample of normal skin which demonstrates abundant TGF-α expression was used as a positive control reference standard for TGF-α expression. Cytospins of A431 (a well-characterized vulvar squamous cell carcinoma cell line which over expresses EGFR) provided as slides containing approximately 25,000 cells per slide were fixed in formaldehyde without saponin and were used as a positive control reference standard for EGFR expression. Negative controls for staining consisted of replacement of the primary antibodies with an isotope matched irrelevant murine IgG subclass antibody.

The intensity of immunochemical staining as a reflection of the number of positive granules per cells (mean labeling concentration=mean optical density) was evaluated under 40× magnification on a SAMBA 4000 Image Analysis System (available from Image Products International of Chantilly, Va.) although other optical assessment systems can substitute. Twelve high power fields of each section were analyzed and the result reported as the mean of the optical density (MOD) of the twelve values. The heterogeneity of staining was also determined by computer analysis and reported as concentration heterogeneity which is defined as the concentration variation coefficient between cells and structure (concentration standard deviation/mean concentration). The samples were coded and the patholo-

gists performing the computerized image analysis were blinded to the clinical outcome of the patients. Human skin samples from three different individuals were stained with TGF-α Ab and analyzed on seven separate occasions to assess the variability of this TGF-α standard. Cytospins of A431 cells were stained for EGFR on four different occasions and expression levels were quantitated to determine the variability of the EGFR standard. The raw data from the tumors were analyzed as a percent of the standards (mean optical density) to control for day-to-day staining variability and to ensure that the results could be generalized for prospective data collection in other laboratories. Human skin samples are readily available in most diagnostic pathology laboratories or are available commercially. A431 cells can be obtained, for example, from the American Tissue Culture Collection, 10801 University Boulevard, Manassas, Va. 20110.

Statistical analysis was performed as follows. Survival was measured in months from the date of surgery to the date of death or to the last follow-up. Disease-free survival was defined as the time from resection until the first evidence of recurrence or the development of a new upper aerodigestive tract primary tumor. All surgical resections were considered curative rather than palliative.

Patients were divided into approximately equal tertiles according to TGF-α and EGFR tumor levels for the purpose of generating survival curves. Survivor function curves and median survival times were calculated according to the methods of Kaplan and Meier, which are well known in the art. Confidence intervals for the median were constructed using Greenwood's formula on the log scale. Differences in survivor function due to prognostic factors were calculated by the log-rank test. P values for multiple log rank tests were adjusted with a step-down Bonferroni procedure. The joint effect of predictive variables was evaluated by Cox regression models using continuous variables. Prognostic covariates included in the analysis were sex, age, tumor site, tumor grade, tumor stage, nodal stage, and the mean optical densities of TGF-α and EGFR protein levels in the tumor. Prognostic factors were evaluated individually and all factors having a moderate or strong impact on survival were considered jointly for Cox regression modeling. To assess reliability of the MOD values, repeated measurements of TGF-α and EGFR were obtained in a subset of samples for each marker and the intraclass correlation was estimated.

The results of the testing and statistical analysis were as follows. All 91 head and neck cancer patients studied expressed TGF-α and EGFR protein in their tumors. The SAMBA 4000 Cell Image Analysis System was used to quantify the intensity of immunostaining. For the positive control skin samples, the mean TGF-α MOD for the positive control samples was 19.26+/−0.99. Due to the low variability in the TGF-α and EGFR standards, the raw data (MOD) was analyzed as a percentage of standard for each measurement. Patients whose tumors expressed high levels of TGF-α protein also expressed elevated levels of EGFR (Spearman correlation=0.70, p=0.0001) and they were more likely to have died of disease than patients whose tumors expressed low levels of TGF-α and EGFR (FIG. 4).

On the basis of TGF-α and EGFR protein expression levels, the 91 patients with head and neck squamous cell carcinoma were considered in tertiles and correlated with clinical and pathological parameters. As shown in FIG. 1, gender, age>65, tumor site, tumor stage, nodal stage, or tumor grade were not significantly associated with TGF-α and EGFR protein expression levels in the primary tumor. The association of clinical and pathologic characteristics of head and neck cancer patients with disease-free survival is shown in FIG. 2. In the univariate analysis, the factors with no significant association with decreased disease-free survival were gender, age>65, tumor grade, tumor stage or nodal stage. Tumor site (larynx; p=0.0414) was associated with increased disease-free survival most likely due to both early diagnosis and the relatively sparse lymphatic drainage of the vocal cords. However, the levels of TGF-α (p=0.0001) or EGFR (p=0.0001) protein expressed in the tumor were the strongest predictors of decreased disease-free survival. Disease-free survival was further examined by censoring either recurrences or second primary tumors. TGF-α and EGFR levels were determined to have a significant impact on the recurrence of the index tumor (p=0.001). Although only seven patients developed second primary tumors during the course of the study, both elevated TGF-α (p=0.0111) and EGFR (p=0.0015) levels in the index tumor were associated with the occurrence of a second upper aerodigestive tract malignancy.

When overall cause-specific survival was examined via univariate analysis, only nodal stage (p=0.0071) TGF-α protein levels (p=0.0001) and EGFR protein levels (p=0.0001) showed significant association with adverse outcome (FIG. 3). Survival curves of patients within each tertile revealed that both TGF-α and EGFR levels in the primary tumor were highly predictive of reduced disease-free ("NED") survival (FIGS. *5a* and *5b*). Both TGF-α and EGFR levels in the primary tumor were also predictive of reduced overall cause-specific survival when divided into tertiles (FIGS. *6a* and *6b*). In a Cox regression model, the combination of TGF-α and EGFR levels plus nodal stage was the strongest predictor of survival. The exclusion of EGFR level (p=0.001) from the model resulted in a significant reduction of predictive power. However, the combination of EGFR levels and nodal stage was as strong a predictor of outcome as TGF-α and EGFR levels plus nodal stage (p=0.13; Cox regression overall cause-specific survival; data not shown).

To determine whether TGF-α and EGFR tumor levels predicted survival independent of nodal metastases, tests of interaction were performed which revealed that the effect of TGF-α and EGFR upon overall survival was the same across N-stage categories (FIGS. *7a, 7b, 7c, 7d*). TGF-α levels were high (MOD>182% of standard) in 12 patients with no evidence of neck metastases ($N_0$) Six of these twelve patients died of their disease during the course of the study. Similarly, 16 patients with clinicopathologic No staging had high EGFR levels (MOD>100% of standard) in their index tumors, 9 of whom subsequently died of disease. Five patients with advanced disease in their neck ($N_2$) had low TGF-α levels (MOD<83% of standard) in the primary tumor and all are still alive without evidence of disease. EGFR levels were low (MOD<23% of standard) in 4 patients with $N_2$ clinicopathologic staging, all of whom are alive without disease. Conversely, TGF-α tumor levels were low in 18 patients, with No staging, none of whom died of disease and EGFR tumor levels were low in 21 patients with $N_0$ staging all of whom remain alive without evidence of disease. Fourteen patients had high TGF-α tumor levels, 11 of whom died of disease and nine patients had high EGFR tumor levels, all of whom subsequently succumbed to their head and neck cancer. These results surprisingly suggest that TGF-α and EGFR expression levels critically either below about 83/23% of standard or, alternatively, greater than 83/23% of standard, give meaningful prediction of clinical outcome even independently of lymph node status and have significant value in the management of head and neck cancer

patients. Because the expression of TGF-α and EGFR has already been documented in other types of malignancies (including bladder, lung, kidney, ovary, brain, cervix, endometrium, esophagus, stomach, pancreas and thyroid), the invention also embraces the method of assessing expression levels according to this important 83/23% of standard threshold, as well.

Expression levels of TGF-α and EGFR mRNA or protein in tissues can be measured by several techniques including radiolabeled ligand binding, Western and Northern blotting, in situ hybridization and quantitative reverse transcription polymerase chain reaction. These molecular techniques, however, are time consuming, requiring a high degree of technical expertise and meticulous processing of the tissue specimen. Also, these techniques are often unable to distinguish the precise cellular source of the molecule(s) under investigation (e.g., tumor cell versus normal epithelial cell versus submucosa). In contrast, immunohistochemistry using commercially available antibodies such as monoclonal antibodies is a standard procedure in all diagnostic pathology laboratories, and can be performed on paraffin-embedded specimens. In the context of the invention, it is both easy and effective to quantify TGF-α and EGFR expression levels in this way. Counting may be manual/optical or standardized optical computer hardware and software may be used, and it was found that even when quantitative image analysis was performed on only a relatively small volume of the tumor tissue, there proved to be a remarkably low level of heterogeneity of either TGF-α or EGFR expression and nothing more was necessary for accurate quantitation.

The easiest way to visualize the criticality of the about <83/23% of standard expression levels of the present invention is to compare FIGS. **5–7** as follows. The "low" expression group in each graph represents the tertile of patients for whom expression levels were lower than about 83/23% of standard. In all but FIGS. **5***a* and **5***b*, the data show lines for the "low" group straight across on the "proportion surviving" axis, i.e., no deaths. Even in FIGS. **5***a* and **5***b*, this same group still had no deaths for at least five years, and the only reason the curve for this group declined somewhat is that a relatively small proportion of this did have some evidence of disease. The "flat lines" of FIGS. **7***a, b, c* and *d* therefore indicate surprising and unexpected results—the expression levels of the present invention are by no means a smooth continuum, but in fact can be seen pictorially to have a critical level with prognostic meaning both above and below. It is the use of this critical level as a threshold, in an otherwise standard laboratory assay thus given entirely new utility, which forms the heart of the present invention,

The alternative way to visualize the unexpected results attributable to the present unexpectedly critical expression threshold is apparent in FIG. **4**. The solid black dots represent deaths from squamous cell head and neck cancer; the stars represent survivors of squamous cell head and neck cancer, and the hollow dots represent deaths from other causes (traffic accidents, etc.), all from the same study of 91 patients. As can be seen dramatically from FIG. **4**, the solid black dots representing deaths cluster within a neat and easily definable sector with respect to elevated TGF-α and EGFR levels, as likewise do the survivor stars. As to the "death" dots, there are not even any statistical "outlyers," which never could have been predicted.

Although the invention has been described with particularity above, with respect to particular methods and patient populations, the invention is only to be limited insofar as is set forth in the accompanying claims.

What is claimed is:

**1**. A prognostic method to predict the survival of a patient already diagnosed to have a malignancy, comprising the steps of:

a) obtaining a sample of malignant cells from a patient;

b) quantifying expression of a protein selected from the group consisting of Transforming Growth Factor Aloha (TGF-α) and Epidermal Growth Factor Receptor (EGFR) in said sample;

c) normalizing the values quantitated in step b) as to standard, to yield a normalized percentage expression as to standard; and

d) assessing whether said normalized percentage expression as to standard falls into the poor or good prognostic category as determined by calculable survival curves by assessing whether said normalized percentage expression as to standard falls into a good prognosis category of less than about 83% of standard for TGF-α or falls into a good prognosis category of less than about 23% of standard for EGFR,

wherein the assessment performed in step d) has prognostic significance in predicting patient survival.

**2**. The method according to claim **1** wherein said malignant cells are selected from the group consisting of squamous cell head and neck cancer cells, bladder cancer cells, lung cancer cells, kidney cancer cells, ovarian cancer cells, brain cancer cells, cervical cancer cells, endometrial cancer cells, esophageal cancer cells, stomach cancer cells, pancreatic cancer cells, and thyroid cancer cells.

**3**. The method according to claim **1** wherein said malignant cells are squamous cell head and neck cancer cells.

**4**. The method according to claim **1** wherein said quantifying is conducted by contacting said malignant cells with monoclonal antibodies specific to TGF-α or EGFR and further quantifying the resulting binding to assess the presence of TGF-α or EGFR.

**5**. The method according to claim **4** wherein said quantifying is conducted on a laboratory slide.

**6**. The method according to claim **5** wherein said quantifying is conducted optically, by eye.

**7**. The method according to claim **5** wherein said quantifying is conducted automatically, using image analysis hardware and software.

**8**. The method according to claim **5**, wherein the results of step d) are considered in association with nodal staging of the patient.

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